Faculty of Resource Science and Technology

Detection and Molecular Characterization of *Bacillus cereus* Isolated from Sago Processing Plants in Sarawak

Jasmin binti Jaraee

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Dr Maurice Bilung
Pensyarah Kanan Industri
Program Biosinologi Sungai
Fakulti Sains dan Teknologi Sumber
Universiti Malaysia Sarawak

Current Address:
132 A, KAMPUNG PANGLIMA SEMAN ULU, 93050, KUCHING SARAWAK.

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Detection and Molecular Characterization of *Bacillus cereus* Isolated from Sago Processing Plants in Sarawak

Jasmin binti Jaraee

A thesis submitted

In fulfilment of the requirements for the degree of Master of Science (Microbiology)

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DECLARATION

The thesis has not been accepted for any degree and is not concurrently submitted in candidature for any other degree.

_____

Name: Jasmin binti Jaraee

Matric number: 15020198

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ABSTRACT

Sago processing industries are well-established and high potential industries in Sarawak. However, the contamination of bacteria might deteriorate its quality and become a concern to the public health. This study aimed to detect, quantify and characterize *Bacillus cereus* in sago processing in Sarawak, Malaysia. *B. cereus* was isolated from two selected sago processing mills in Sarawak. The prevalence and concentration of *B. cereus* in this study were determined firstly using selective agar and followed by using specific Polymerase Chain Reaction (PCR) by targeting specific virulence gene, haemolysin (*hly*) gene. A total of 120 samples consist of bark swab, sago pith, starch slurry, sago milk, sago flour and sago effluent were collected from each processing step in sago mills. It was revealed that *B. cereus* were present in 35% (42/120) of the samples. These isolates were subjected to molecular typing by using Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC PCR) and Pulsed Field Gel Electrophoresis (PFGE). Both molecular typing method showed heterogeneity of the *B. cereus* strain. Susceptibility of all isolates towards 14 antibiotics was assessed using disk diffusion assay. *B. cereus* isolates were uniformly resistant to penicillin and ampicillin whereas *B. cereus* isolates were uniformly susceptible to imipenem and norfloxacin. Multiple antibiotic resistance (MAR) index were calculated based on the antibiotic resistance results. The MAR index for all isolates were also varies, ranged from 0.083 to 0.750. This study is useful in developing appropriate intervention strategies and establishing food safety standards in sago processing in Sarawak thus contribute in lowering the disease burden and assist in providing safer food to the society.

Keywords: *B. cereus*, sago processing, detection, polymerase chain reaction, molecular characterization, antibiotic susceptibilities test

Pengesanan dan Pencirian Molekul Bacillus cereus yang Diasingkan dalam Kilang Pemprosesan Sagu di Sarawak

ABSTRAK

Industri pemprosesan sagu merupakan industri yang dikenali dan berpotensi tinggi di Sarawak. Walau bagaimanapun, pencemaran bakteria akan mengurangkan kualitinya dan menjadi isu kepada kesihatan awam. Kajian ini bertujuan untuk mengesan, mengira dan mencirikan B. cereus dalam pemprosesan sagu di Sarawak. B. cereus diasingkan daripada dua kilang pemprosesan sagu yang terpilih di Sarawak. Kelaziman dan penumpuan B. cereus dalam kajian ini dikaji dengan menggunakan agar selektif dan kemudian diuji dengan reaksi polymeras berantai spesifik untuk mengekspresikan gen sasaran iaitu gen hemolaisin. Sejumlah 120 sampel yang terdiri daripada olesan batang, empulur sagu, buburan kanji, susu sagu, tepung sagu dan efluen sagu diperolehi daripada setiap peringkat pemprosesan kedua-dua kilang. Ia menunjukkan bahawa B. cereus wujud di dalam 35% (42/120) sampel. Isolat ini tertakluk kepada pencirian molekul dengan menggunakan ERIC-PCR dan PFGE. Hasil daripada kedua-dua pencirian molekul ini menunjukkan kepelbagaian jenis B. cereus. Sensitiviti kesemua isolat terhadap 14 antibiotik ditaksir menggunakan B. cereus menunjukkan kerintangan kepada penisilin dan ampisilin serta sensitif kepada imipenum dan norfloxazin. Kajian ini berguna dalam membangunkan strategi untuk langkah pembaikan serta menghasilkan piawaian keselamatan makanan dalam pemprosesan sagu di Sarawak sekaligus menyumbang dalam mengurangkan beban penyakit dan membantu ke arah penyediaan makanan yang selamat kepada masyarakat.

Kata kunci: *B. cereus, pemprosesan sagu, pengesanan, tindak balas polimeras berantai, pencirian molekul, ujian sensitiviti antibiotik.*

TABLE OF CONTENTS

LIST OF TABLE

LIST OF FIGURES

LIST OF ABREVIATIONS

µL	Microliter
µm	Micrometer
ºC	Degree Celsius
%	Percent
AFLP	Amplified fragment length polymorphism
AGE	Agarose gel electrophoresis
AOAC	Association of Official Analytical Chemists
AST	Antibiotic susceptibility test
CDC	Centre for Disease Control
CFU	Colony forming unit
ddH_2O	Double distilled water
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
ERIC	Enterobacteria repetitive intergenic consensus sequences
EtBr	Ethidium bromide
FDA	Food and Drug Association
FSIS	Food Safety and Inspection Service
ISO	International Organization of Standardization
HBL	Haemolysin BL
kb	Kilo base
kDa	Kilo Dalton

MAR	Multiple antibiotic resistant
$MgCl_2$	Magnesium Chloride
mL	Milliliter
MLST	Multilocus sequence typing
Mm	Milimeter
mM	Milimolar
MPN	Most probable number
MSRV	Modified Semisolid Rappaport-Vassiliades
MYP	Mannitol-Egg Yolk-Polymyxin
NCBI	National Center for Biotechnology Information
n.d	No date
NGFIS	Netherlands Government Food Inspection Services
NHE	non-hemolytic enterotoxin
NMKL	Nordic Committee on Food Analysis
PCR	Polymerase chain reaction
PEMBA	Polymyxin pyruvate egg-yolk mannitol–bromothymol blue agar
PFGE	Pulsed-field gel electrophoresis
RAPD	Random amplification of polymorphic DNA
rep-PCR	Repetitive Polymerase Chain Reaction
RM	Ringgit Malaysia
Rpm	Revolution per minute
RTE	Ready-to-eat
SLST	Single locus sequence typing

U	Unit
UV	Ultra violet
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA
TBS	Tryptone soy broth
USDA	United States Department of Agriculture
VNTR	Variable number tandem repeat
WHO	World Health Organization

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

In Sarawak, sago processing industries is a well-established industry that contribute to Sarawak export revenue (Karim *et al.*, 2008). Sago palm is the plant that has high potential agricultural industry and is grown commercially in Southern East Asia countries (Singhal *et al.*, 2008). According to Adeni *et al.* (2009), sago is very important food product for over a million peoples as their primary dietary starch source. Starch is one of the most abundant plant products in the world. It is a major source of energy in human daily food. Every year, the consumption of the sago starch by the global citizen is estimated to be between 200,000 to 300,000 tons (Bujang and Muniandy, 2004). The export of sago product from Sarawak in 2013 was estimated to be 50,000 tonne which procuring income approximately RM 81 millions (Department of Agriculture Sarawak, 2013). Usually, sago starch is used for various applications in Malaysia such as glucose, monosodium glutamate and noodles (Bujang, 2008).

1.1.1 Problem Statement

In food processing industries, some of the preparation, processing and storage procedures were exposed to the risk of bacterial contamination. It might harbor a great number of microorganisms that may cause the food products to spoil and represents a direct health hazards to consumers (Lesley *et al.*, 2013). Most of the incidence of diarrhoea in developing countries are caused by foodborne and waterborne pathogens (WHO, 2008). Diarrhoeal diseases are included in 20 leading causes of death in the world in 2014 (WHO, 2014). Unhygienic food handling and preparation and inadequate cooking which may lead to food

poisoning due to the presence of bacteria. (Sandra *et al*., 2012). Until March 2015, 143 cases of food poisoning were reported in Sarawak (Sarawak EPID Health News, 2015). There are a few food poisoning bacteria reported to be commonly found in food and its processing which include *Salmonella* spp., *B. cereus, Escherichia coli* and *Listeria* species (Greenhill *et al.,* 2007).

B. cereus is chosen as potential hazard due to its ubiquitous nature and its preference to live in soil and starchy food. The presence of the *B. cereus* in food processing industries cause problems by reducing the quality of the products (Lesley *et al.,* 2013; Sandra *et al.,* 2012), and affecting people's health after eating the contaminated foods (Ghelardi *et al.,* 2002). *B. cereus* was first discovered by Frankland *et al*. (1887) in the air of the cowshed. *B. cereus* affects foods processing industry both by reducing the quality of the products (Lesley *et al.,* 2013; Sandra *et al.,* 2012) and by endangering people's health upon eating contaminated foods (Ghelardi *et al.,* 2002).

However, to date, there is limited study of the occurrence of microorganisms including *B. cereus* in sago-associated products has been conducted (Greenhill *et al.,* 2007). To the best of our knowledge, there has been no study on the prevalence of *B. cereus* in sago processing has been reported in Sarawak. Hence, in our study we isolated and identified *B. cereus* from sago processing and determine the prevalence of the *B. cereus.* There are several international standard protocol commonly used for isolation and identification of *B. cereus* which include the US Food and Drug Administration (FDA), the Association of Official Analytical Chemists (AOAC), the European Committee for Standardisation (CEN, EN), the International Organization for Standardization (ISO), the Netherlands Government Food Inspection Services (NGFIS), the Nordic Committee on Food Analysis (NMKL) and the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS). In our study, we applied

FDA method which involve the sample enrichment by using tryptone soy broth and direct plating on MYP agar (Lesley *et al.*, 2013).

B. cereus is further identified and confirmed by PCR assay. Further confirmation and identification by PCR assay were required as conventional detecting method often give false-positive/false-negative results. Hence PCR assay can be applied as it is reliable, specific, sensitive and reproducible method. In the present study, we detect *B. cereus* by targeting haemolysin gene as described by Mutashar *et al.* (2015), Fukushima *et al.* (2003) and Wang *et al*. (1997). The raw material quality and the processing environment of food products can affect the final products quality (Oh *et al.*, 2012). As contamination and/or cross-contamination were the major concern in food processing, hence we decided to conduct molecular typing of *B. cereus* by using rep-PCR method in order to determine the contamination pattern in the sago processing mills. It is important to understand the routes of contamination of *B. cereus* in sago processing to prevent the contamination of the final products (Oh *et al.*, 2012).

B. cereus is an opportunistic pathogen that can cause severe foodborne illness. There were also several studies on the resistance of *B. cereus* toward antibiotics had been reported (Yim *et al.*, 2015; Lee *et al*., 2012; Park *et al.*, 2009; Rosenquist *et al.*, 2005 and Andrews *et al.*, 2002). This showed it is necessary to investigate the *B. cereus* antibiotic resistance and susceptibility. Antibiotics have been widely used in agriculture, veterinary and medicine. Prolonged usage and exposure towards commonly used antibiotics including penicillin, ampicillin, norfloxazin, imipenem, tetracycline, chloramphenicol, quinolones, aminoglycoside, spectinomycin, cephalosporin, nitrofuran, nitromidazole, sulfonamide, and trimethoprim are able to promote the emergence of resistance in bacteria. It is important to monitor antibiotic susceptibility of *B. cereus* in food processing to ensure and monitor the emergence and spread of bacterial resistance to antimicrobial agents. However, there is no data of antibiotic resistance

of bacteria associated with sago processing mills. In this study, the antibiotic susceptibility of *B. cereus* in the sago was examined by disk diffusion method (Park *et al.,* 2009). The findings of this study provided baseline data on *B. cereus* contamination for future risk assessment work and establishment of food safety standards in sago processing in Sarawak.

1.1.2 Objectives

The main goal of this study was to detect, quantify and characterize *B. cereus* in sago processing in Sarawak, Malaysia. The specific objectives of this study were to:

a. Detect *B. cereus* in sago processing by using Polymerase Chain Reaction.
b. Determine the contamination level of *B. cereus* in sago processing by enumerating *B. cereus* using standard plate count method.
c. Determine the antibiotic resistance profiles of *B. cereus* isolated from sago processing.
d. Characterise *B. cereus* in sago processing by using Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC- PCR) and Pulsed-field Gel Electrophoresis (PFGE).

1.2 Literature review

1.2.1 Sago palm

Sago palm (Figure 1.1) or *Metroxylon sagu* belongs to the genus *Metroxylon* of Palmae family (Greenhill *et al.,* 2007). *Metroxylon sagu* is derived from the term 'metra' which means pith or parenchyma and the term 'xylon' means xylem. Many scientists considered it as the starch crop of the 21st century (Singhal *et al.,* 2008). It is due to its ability to store and yield more starch in its stem than that of any other starch crop (Onsa *et al.,* 2004). *Metroxylon sago* is able to survive swamp area, acidic peat soils and in high saline area (Ehara *et al.,* 2000). It needs little management and able to live in marginal agricultural land (Purwanto *et al.,* 2002).

The plant is believed to have originated from Papua New Guinea and/or Moluccas where Papua New Guinea considered as the center of the diversity of sago palm (Toyoda, 2008). In Malaysia, Sarawak is the largest area for the *Metroxylon sagu* plantation which is approximately 55500 hectares in 2012 (Department of Agriculture Sarawak, 2013). Seventy-five percent of Sarawak sago planting area are found in Mukah (Bujang, 2010).

Figure 1.1: Sago plantation in Mukah, Sarawak (Bujang, 2008).

1.2.2 Sago processing

Sago was processed commercially using the conventional and modern method in Sarawak (Shin and Collins, 2015). Conventional method and modern method had been described by Shin and Collins (2015) and Bujang (2008), respectively. Modern method usually being practiced in sago processing mills. In the modern method, mature sago trunk was selected and cut. After sago trunk is cut, it will be further cut into smaller one meter logs (Figure 1.2). The smaller logs of sago trunks will be transported to the sago processing mill by using lorries

or tied to form rafts so that they can be transported via river to the mill (Figure 1.3). Then, the bark of the sago trunk will be debarked manually or using automated debarking machine (Figure 1.4). The debarked logs are mashed using a rasper, followed by a hammer mill and then water is added to form starch slurry (Figure 1.5). Then, the starch slurry is allowed to settle down in the tank, dry and finally form sago flour that are ready for packaging (Figure 1.6). Figure 1.7 shows the schematic flow diagram of the sago processing.

Figure 1.2: Sago trunk was selected before cut into smaller 1 meter log.